

In the Specification:

The paragraph starting at page 3, line 23 has been amended as follows:

--The most potent aptamer, TIP 12/1 (SEQ ID NO: 3), showed a similar binding affinity for mdm2 as bacterial full length w p53. This made it a powerful inhibitor which could be expressed in mammalian cells.--

The paragraph starting at page 6, line 10 has been amended as follows:

--Figure 3: Soluble β -galactosidase assays of cell lysates transfected with RGC Δ lacZ and TIP 12/1 (SEQ ID NO: 3) (black bars) or Trx (white bars) encoding DNA. The highest activity was measured in MCF-7 cells transfected with TIP 12/1 (SEQ ID NO: 3) and set 100%.--

The paragraph starting at page 7, line 9, has been amended as follows:

--Variant peptides have an amino acid sequence which differs from wt p53 sequence, e.g. in the motif between amino acids 13-41 described in WO 96/02642, by one or more of addition, substitution, deletion and insertion of one or more amino acids, but which retains the activity of binding to mdm2. Such variants preferably include the motif FxxxW (SEQ ID NO: 4), where x is any amino acids, and will typically share at least about 70%, more preferably at least about 80%, more preferably at least about 90%, or more preferably at least about 95% amino acid sequence identity with the corresponding portion of human p53. Examples of peptides capable of disrupting the interaction of p53 and mdm2 ~~and~~ are the thioredoxin insert peptides (TIPs) disclosed in Böttger et al, 1996, and in the examples below, see especially peptide TIP 12/1 (SEQ ID NO: 3).--

The paragraph starting at page 21, line 5 has been amended as follows:

--For cloning of TIP 12/1 (SEQ ID NO: 3), TIP wt (SEQ ID NO: 2) and Trx into pcDNA3 for expression in mammalian cells, the thioredoxin coding region complete with the peptide insertions, was amplified from pTrx, pTrx 12/1 and pTrx wt using the following primers:--

The paragraph starting at page 21, line 12 has been amended as follows:

--The resulting PCR products were cleaved with BamHI and Eco RI and ligated into BamHI, EcoRI cleaved pcDNA3. The TIP 12/1 (SEQ ID NO: 3) sequence in pcDNA3 was verified by sequencing.--

The paragraph starting at page 21, line 30 has been amended as follows:

--For microinjection, cells were seeded onto tissue culture dishes and grown to 60-70% confluence. Microinjection was performed using an Eppendorf microinjection system (Microinjector 5242, Micromanipulator 5170) mounted to an Axiovert 35 M with heated stage. Antibody injections were intranuclear or cytoplasmic. Plasmid injections were intranuclear. Purified mouse monoclonal antibodies 3G5 and 4B2 were injected in PBS at a concentration of ca 1.3 mg/ml. Plasmid DNA encoding for TIP 12/1 (SEQ ID NO: 3), TIP (SEQ ID NO: 2) and Trx was purified using Quiagen purification system or phenol/chloroform precipitation and injected at a concentration of 0.25 mg/ml in water. Following microinjection fresh medium was added to the cell cultures and they were incubated for 24 hours.--

The paragraph starting at page 22, line 35 has been amended as follows:

--Cells were seeded into 6 well plates at 1.5×10^6 cells per well. They were grown to a density of 80% confluence and transfected using different lipophilic reagents (lipofectin and lipofectamin, Promega, DOSPER and DOTAP, Boehringer). 2.5 μ g TIP (SEQ ID NO: 2) encoding plasmid DNA and 1 μ g RGC Δ FosLacZ DNA and 5-10 μ g of lipophilic reagent according to the instructions of the manufacturers were mixed in serum free medium and applied to the cells. 2-4 hours after transfection complete medium was added. 48 hours after transfection β -galactosidase activity was measured using CPRG (Boehringer) as a substrate. Cells were scraped into PBS and centrifuged. Pellets from each well were dissolved in 50 μ l Reporter Lysis buffer (Promega) and incubated on ice for 15 min. Soluble lysates were incubated with CPRG in 100 mM phosphate buffer, pH 7.0. OD at 595 nm was measured 1-24 hours later. To measure transfection efficiencies in each experiment, cells in a separate well were transfected with 2.5 μ g pG3 DNA, encoding firefly luciferase. Luciferase activity was measured using Promegas luciferase assay system. The same lysates served as control for endogenous β -galactosidase activity.--

The paragraph starting at page 24, line 27 has been amended as follows:

-- Figure 1 shows a schematic representation of the three aptamers we constructed by inserting additional peptide sequences into the active site of *E. coli* thioredoxin. TIP 12/1 (Thioredoxin Insert Protein, SEQ ID NO: 3) contains the sequence we identified previously by phage display as the most potent inhibitor of the mdm2-p53 interaction in *in vitro* assays (Böttger et al, 1996). TIP wt (SEQ ID NO: 2) contains the sequence corresponding to p53 wild type sequence P¹³ to N²⁹. As controls we expressed thioredoxin lacking a peptide insertion (Trx) in bacteria.--

The paragraph starting at page 25, line 16 has been amended as follows:

--1. TIP 12/1 (SEQ ID NO: 3) inhibits the interaction between p53 and mdm2 in this assay with the same strength as full length p53. This should make it a suitable agent to be tested in cellular systems for effects on the interaction between p53 and mdm2 *in vivo*.--

The paragraph starting at page 25, line 21 has been amended as follows:

--2. TIP wt (SEQ ID NO: 2) inhibits the interaction 20 times less than TIP 12/1 (SEQ ID NO: 3). This has to be attributed to the 50 times less potent inhibition achieved by the wt peptide when compared with peptide 12/1 in peptide competition assays (Böttger et al, 1997).--

The paragraph starting at page 25, line 31 has been amended as follows:

--In these *in vitro* assays, TIP 1/2 (SEQ ID NO: 3) exhibits strong enough inhibitory potential to compete against endogenous levels of wt p53 in tumor cells for binding to mdm2. It therefore offers an agent that should be capable to function inside mammalian cells.--

The paragraph starting at page 26, line 2 has been amended as follows:

-- We therefore went on to clone TIP 12/1 (SEQ ID NO: 3), TIP wt (SEQ ID NO: 2) and Trx into pcDNA3 (Promega), a vector where these proteins would be expressed under the control of the strong CMV promoter in mammalian cells.--

The paragraph starting at page 26, line 24 has been amended as follows:

--In these experiments we found the strong induction of β -galactosidase activity after microinjection of 3G5 into the nuclei of VRn.6 cells. To be certain that the cells which responded with β -galactosidase activity were the injected ones we carried out dual immunofluorescence studies. We stained cells after injections with anti β -galactosidase antiserum and with anti mouse immunoglobulin. This showed that the cells expressing the reported enzyme (left hand panel, blue) are also positive for the injected antibody (right hand panel, green). Injecting TIP 12/1 (SEQ ID NO: 3) encoding plasmid into the nuclei of Vrn.6 cells also has a strong effect on induction of the reporter enzyme. There is no induction after microinjection of the control thioredoxin encoding plasmid, although it clearly is expressed.--

The paragraph starting at page 27, line 2 has been amended as follows:

--The remarkable strength of this response lead us to test whether this was due to the relatively high levels of p53 and mdm2 present in the CRn.6 line. To do this the microinjections experiments were then carried out in T22 cells, mouse prostate cell derived cell line, also stably transfected with the same reporter plasmid. These cells normally contain very low levels of p53 and mdm2. On treatment with DNA damaging agents the p53 protein accumulates and the cells show remarkable p53-dependent β -galactosidase induction (Hupp et al, 1995, Lu and Lane, 1993). On microinjection of 3G5 and TIPs encoding plasmids into T22 cells, we again detect immense induction of β -galactosidase with 3G5, but no induction with 4B2, an anti mdm2 antibody that targets an epitope outside the p53 binding pocket on mdm2. A remarkable reporter induction was caused by our strongest aptamer, TIP 12/1 (SEQ ID NO: 3). β -galactosidase activity are observed with TIP (SEQ ID NO: 2) and no activity with the control thioredoxin. This reflects exactly the capacity of the three aptamers to inhibit the mdm2-p53 interaction in vitro. Staining of the injected cells with anti-thioredoxin antibody confirmed that the differences in reporter enzyme activity were not due differential expression of the TIP proteins.--

The paragraph starting at page 27, line 32 has been amended as follows:

--We ought to analyse more cell lines, preferentially not dependent on a stably integrated reporter plasmid. We therefore transiently transfected cells containing wt p53 with p53 responsive reporter plasmids (RGCA Δ FosLacZ) and TIP 12/1 (SEQ ID NO: 3) or Trx control. We

choose OSA cells, a human osteosarcoma cell line (Florenes et al, 1994) as an example for a cell line with highly elevated mdm2 levels due to gene amplification. We also used U2-OS cells, another osteosarcoma cell line, which has no gene amplification for mdm2 but elevated levels of mdm2-mRNA (Florenes et al, 1994) and NCF-7 cells, a breast cancer cell line with heterogeneously expressed low levels of wt p53 and no reported mdm2 elevation. First we tried to analyse the protein levels expressed in these cells and the degree of complex formation with mdm2, using immunoprecipitations. We precipitated from cell lysates with the monoclonal anti-mdm2 antibody 4B2 and the monoclonal anti-p53 antibody 421. Figure 2 shows the results.--

The paragraph starting at page 28, line 31 has been amended as follows:

--We then cotransfected (RGCFosLacZ) reporter plasmid and TIP 12/1 (SEQ ID NO: 3) or Trx encoding DNA into all three cell lines to compare the levels of reporter enzyme induction directly. Different lipophilic transfection agents were used and the transfection efficiency was monitored on separate plates by transfecting pG3, a plasmid encoding firefly luciferase under a constitutive promoter, and measuring luciferase activity. This allowed us to exclude the possibility that the difference in p53 dependent transcriptional activation of the reporter are related to transfection efficiency (data not shown). Figure 3 shows an average of four experiments of induction of β -galactosidase activity by TIP 12/1 (SEQ ID NO: 3), compared to Trx in each cell line.--

The paragraph starting at page 29, line 8 has been amended as follows:

--Surprisingly, most induction of the p53 reporter is achieved by TIP 12/1 (SEQ ID NO: 3) in MCF-7 cells and in U2-OS cells, where the level of mdm2 is below the detection limit. A ca. 100 times lower effect of reporter enzyme induction TIP 12/1 (SEQ ID NO: 3) is observed in OSA cells. Figure 3 also shows, that transfection of control plasmid alone induces a low level response of p53 dependent transcriptional activation in MCF-7 and U2-OS cells. This effect has been reported before (Renzig and Lane, 1993). It is, however, almost completely absent in OSA cells.--

The paragraph starting at page 29, line 30 has been amended as follows:

--The dramatic induction of the p53 response by UV and other genotoxic agents is accompanied by the accumulation of high levels of p53 protein due at least in part to its extended half life in treated cells. However, it is not clear that this is the sole mechanism by which the p53 response is activated, ~~an~~ other mechanisms such as allosteric activation of DNA binding function have also been proposed to play a role (Hupp et al., 1995). The very strong induction of the p53 response by the TIP 12/1 (SEQ ID NO: 3) encoding plasmid and PAb 3G5 injection lead us to ask if this induction occurred independently of the accumulation of p53 protein. To test this hypothesis, Mabs 3G5 and 4B2 and TIP 12/1 (SEQ ID NO: 3) and Trx encoding plasmids were injected into T22 cells and the levels of p53 analysed in immunofluorescence. We found that p53 accumulates to high levels in the cells injected with the interaction disrupting 3G5 antibody, but not with the control anti-mdm2 antibody. In a similar way, cells injected with the TIP 12/1 (SEQ ID NO: 3) expression plasmid accumulate high levels of p53, whereas those injected with control plasmid do not. These striking results demonstrate that disruption of the p53-mdm2 interaction mirrors the genotoxic response not only by activating p53 dependent transcription, but also by leading to the accumulation of p53 protein. The implication of these results was that in normal cells mdm2 targets p53 for destruction. When we transfected wild type p53 into p53 negative SAOS 2 cells we found that cotransfection of an mdm2 expression plasmid greatly reduced the level of p53 that accumulated (Figure 4, lanes 2 and 3), supporting the idea that mdm2 could target p53 for degradation. To further test this hypothesis we constructed a point mutant F¹⁹→A in murine p53 that mutated one of the key contact residues of the p53-mdm2 interface identified in the crystal structure and in our phage display analysis (Böttger et al, 1997; Kussie et al., 1996). we confirmed that this mutant p53 was unable to bind o mdm2 but was transcriptionally active (data not shown). When this mutant p53 was transfected into the SAOS 2 cells its accumulation unlike that of wild type p53 was not affected by cotransfection of the mdm2 expression plasmid (Figure 4, lanes 4 and 5).--

The paragraph starting at page 30, line 37 has been amended as follows:

--The above results show that peptide aptamers able to block the binding of p53 to mdm2 in cellular assays. To do this peptide sequences capable of binding tightly to mdm2 that has been identified from phage peptide libraries were displayed on the active site loop of thioredoxin. The aptamer proteins (TIPs) were readily expressed in *E. coli* and easily purified from soluble

lysates. In *in vitro* assays the peptides inserted into the active site loop of thioredoxin showed the same greatly enhanced binding compared to the wild type p53 peptide sequence that we had seen in our earlier analysis of the free peptides, demonstrating the successful transfer of the optimized sequence from phage display to insert protein. Critically, the inhibitory potential of the 15 kD TIP 12/1 (SEQ ID NO: 3) protein was the same as that of tetrameric wt p53 and greatly exceeded that achieved by the simple transplantation of the wild type p53 sequence into thioredoxin. When expressed in mammalian cells both aptamers, TIP 12/1 (SEQ ID NO: 3) and TIP wt (SEQ ID NO: 2) were able to induce p53 dependent transcriptional activation of a reporter gene. The intensity of the effect in microinjection experiments exerted by TIP 12/1 (SEQ ID NO: 3) in comparison with TIP wt (SEQ ID NO: 2) was proportional to their *in vitro* inhibitory potential. With these mdm2 binding aptamers we therefore have developed powerful tools to study the biological consequences of disrupting the interaction between mdm2 and p53 in tumour cells. This precise approach of aptamer selection and design describes a road leading from the identification of peptides that are able to disrupt a very specific protein-protein interaction by combinatorial library approaches towards verifying the expected biological effect *in vivo*. This concept in combination with the use of peptide aptamer libraries, for instance expressed and screened the yeast two hybrid system (Colas et al, 1996) should have a great potential for studying networks of protein-protein interactions in eucaryotic cells on the biochemical as well as on the functional level. It certainly now enabled us to draw some interesting conclusions about the significance of inhibiting the interaction between mdm2 and p53 in tumour cells.--

Table 1 on page 35 has been amended as follows:

Inhibitor	IC ₅₀ in nM
TIP wt (<u>SEQ ID NO: 2</u>)	15 000
TIP 12/1 (<u>SEQ ID NO: 3</u>)	300
Trx	no inhibition
peptide wt	2 000
peptide 12/1	100
full length p53	400